

**Method for investigating cytosine methylation in DNA sequences by means of  
hemi-methylation sensitive restriction enzymes**

The present invention concerns a method for investigating cytosine methylations in DNA sequences.

**Background of the invention**

5-Methylcytosine is the most frequent covalently modified base in the DNA of eukaryotic cells. It plays an important biological role, among other things, in the regulation of transcription, in genetic imprinting and in tumorigenesis (for review: Millar et al.: Five not four: History and significance of the fifth base. In: The Epigenome, S. Beck and A. Olek, eds.: The Epigenome. Wiley-VCH Publishers, Weinheim, 2003, pp. 3-20). The identification of 5-methylcytosine as a component of genetic information is thus of considerable interest. A detection of methylation is difficult, of course, since cytosine and 5-methylcytosine have the same base pairing behavior. Many of the conventional detection methods based on hybridization thus cannot distinguish between cytosine and methylcytosine. In addition, the methylation information is completely lost in a PCR amplification.

The usual methods for methylation analysis operate essentially according to two different principles. In the first, a selective chemical conversion of unmethylated cytosines to uracil (bisulfite treatment) is employed, and in the second, methylation-specific restriction enzymes are used. The DNA that has been pretreated enzymatically or chemically is then amplified for the most part and can be analyzed in different ways (for review: WO 02/072880, pp. 1 ff).

The conventional methods suffer from several disadvantages. For example, bisulfite treatment takes time and is labor-intensive. There is also the possibility that the DNA is only incompletely converted and also that it is partially degraded. Quantification is difficult both for the chemically as well as the enzymatically pretreated DNA. An amplification is necessary for this purpose, for the most part a PCR. This additional working step is associated with several problems, e.g. the risk of a preferred

amplification of specific sequences (so-called "bias").

The method according to the invention, in contrast, does not require an amplification and thus permits a more rapid and simpler analysis than the conventional methodology. A quantification is also made possible. In the method according to the invention, the DNA to be investigated is hybridized with oligonucleotides of a defined methylation state. In this way, depending on the methylation status of the DNA to be investigated and on the oligonucleotides employed, hybrids are formed, which possess either the same or a different methylation state on the two DNA strands. Subsequently, the hybrids are reacted with restriction enzymes, wherein the restriction is dependent on the methylation state of the hybrids. Then the methylation status of the DNA can be concluded by means of various possible detection techniques.

A hybrid formation and a subsequent, different restriction of the differently methylated hybrids is also utilized in the case of so-called Genomic Mismatch Scanning (GMS). This involves a method for the detection of polymorphisms. Here, the DNA strands of two different individuals are hybridized with one another, wherein the DNA of one individual has been synthetically methylated beforehand with the use of enzymes. The homohybrids, that are formed are then digested by enzymes, while the heterohybrids are further analyzed (see, e.g.: Nelson et al.: Genomic mismatch scanning: a new approach to genetic linkage mapping. Nat Genet. 1993 May; 4 (1) :11- 8). The synthetic methylation and the subsequent restriction thus are used here for the isolation of heterohybrids. A utilization of the different restriction of differently methylated DNA hybrids for the quantitative analysis of natural DNA methylation patterns has not been described previously.

### Description

In the method according to the invention, the DNA to be investigated is hybridized with oligonucleotides of a defined methylation status and then reacted with specific restriction enzymes. The restriction enzymes are able to recognize CpG sequences and also to distinguish hemi-methylated DNA double strands either from unmethylated

or from homo-methylated DNA double strands. These restriction enzymes are denoted in the following as *hemi-methylation-sensitive*. The terms *hemi-methylated*, *homo-methylated*, *unmethylated* and *defined methylation status* are to be understood as follows: If the cytosines are methylated in the CpG position to be investigated, then they form double strands with oligonucleotides whose corresponding CpG position is unmethylated, and in these double strands, this special CpG position is methylated only on one strand (hemi-methylated). The same applies to hybrids of the corresponding methylated oligonucleotides and unmethylated DNA to be investigated. In contrast, if both the oligonucleotide as well as the DNA are methylated at the CpG position, then a methylated double strand results on both sides (homo-methylation). And vice versa, if the CpG positions both in the DNA to be investigated as well as also in the oligonucleotide are unmethylated, then unmethylated double strands are formed. The terms hemi-methylated, homo-methylated and unmethylated thus do not describe the total methylation state of the DNA in the following, but only the state of individual CpG positions within the DNA. Defined methylation status is to be understood in that the cytosines of the oligonucleotides utilized are either unmethylated or methylated at the 5-position in the CpG positions which correspond to the CpG positions to be investigated in the DNA.

The method for the methylation analysis according to the invention is comprised of the following four steps:

- a) the DNA to be investigated is hybridized to oligonucleotides of a defined methylation status.
- b) the hybrids are reacted with at least one hemi-methylation-sensitive restriction enzyme,
- c) a detection is made of whether a restriction has occurred,
- d) the methylation state of the investigated DNA is concluded.

In the first step of the method according to the invention, the DNA to be investigated is

hybridized to oligonucleotides. This can be conducted both in solution as well as to a solid phase. The DNA to be investigated can originate from different sources. For diagnostic purposes, tissue samples, among others, can be used as the initial material, but body fluids, particularly serum, can also be used. It is also conceivable to use the DNA from sputum, stool, urine, or cerebrospinal fluid. Preferably, the DNA is isolated from the biological specimens. The DNA is extracted according to standard methods, from blood, e.g., with the use of the Qiagen UltraSens DNA extraction kit. The isolated DNA is then fragmented, e.g., by reaction with conventional (not hemi-methylation-sensitive) restriction enzymes. The reaction conditions and the enzymes employed are known to the person skilled in the art and result, e.g., from the protocols supplied by the manufacturers.

The oligonucleotides at the cytosine positions which are recognized by the restriction enzyme that will be used later are unmethylated or methylated at the 5-position, depending on the restriction enzyme that will be used. For a quantitative analysis, both methylated as well as unmethylated oligonucleotides are utilized (see below). The synthesis of correspondingly unmethylated and methylated oligonucleotides is part of the prior art. In another preferred variant, several oligonucleotides of different sequence are used, so that an investigation of several methylation positions simultaneously is possible.

In a preferred embodiment, the oligonucleotides also bear at least one detectable label. A plurality of possible labels is known to the person skilled in the art. Thus, e.g., dyes, fluorescent labels, radionuclides, electrical charge carriers or labels that can be detected in the mass spectrometer are utilized. Peptide labels are also conceivable, which are detected indirectly by the binding of another labeled antibody. Chemical labels are also possible, which are made visible by subsequent reaction with another labeled marker molecule. Many other labeling possibilities also belong to the prior art. Preferably, the oligonucleotides of different sequence or different methylation status bear different labels.

In a particularly preferred variant, the oligonucleotide bears a fluorescent dye on one

side of the restriction site and a so-called "quencher" on the other side. If a restriction is produced, then the dye and the quencher will be separated, so that the dye signal can be detected (Fig. 1). Dyes and quenchers that can be used are known to the person skilled in the art.

The oligonucleotides are preferably bound to a solid phase. The type of solid phase and the solid phase coupling are prior art. For example, solid phases can involve functionalized polymers, metals, glass or semiconductors such as silicon. The oligonucleotides can be bound e.g., via bifunctional linker molecules, which are bound to a silanized surface or, for example, via thioates or thiol modifications in the oligonucleotide to bromoacetyl [acetyl bromide]-derivatized surfaces or gold. The oligonucleotides of different sequence or different methylation status are preferably spatially separated from one another so that a separate detection is possible.

In another preferred embodiment, the oligonucleotides are introduced onto a sensitive surface, whose physical or chemical properties can be modified in a measurable way by means of a restriction. For example, conductivity, characteristic frequency or surface tension are such measurable properties. In a particularly preferred variant, this surface is comprised of a piezoelectric crystal. The binding of DNA to piezoelectric crystals is known to the person skilled in the art (for review: Skladal: Piezoelectric Quartz Crystal Sensors Applied for Bioanalytical Assays and Characterisation of Affinity Interactions. J. Braz. Chem. Soc., Vol.14, No.4, 491-502, 2003).

The hybridization of the oligonucleotides to the DNA to be investigated is performed under standard conditions.

In the second step of the method according to the invention, the hybrids are reacted with hemi-methylation-sensitive restriction enzymes. The selection of the restriction enzymes is made according to the sequence specificity of the enzymes and according to the diagnostic or scientific objective to be investigated. Enzymes which cleave unmethylated and hemi-methylated DNA preferably as opposed to homo-methylated DNA are preferably utilized. If one starts with methylated oligonucleotides in this case,

then methylated and unmethylated cytosine positions can be different in the DNA to be investigated. The methylated DNA forms homo-methylated hybrids with the methylated oligonucleotides, and these hybrids are not cleaved by the restriction enzyme. In contrast, in the case of unmethylated DNA, hemi-methylated hybrids are formed, which are recognized by the restriction enzyme and are reacted. On the other hand, if one starts with unmethylated oligonucleotides, then hemi-methylated hybrids are formed with the methylated DNA and unmethylated hybrids with unmethylated DNA. Both hybrids are cleaved by the enzyme, so that a differentiation between methylated and unmethylated DNA is not possible in this case with the use of unmethylated oligos. The use of unmethylated oligonucleotides in addition to the use of methylated oligonucleotides of the same sequence, however, permits a quantification of the method. The ratio between total DNA and methylated DNA can be calculated from the ratio of the two signals. Such a quantification is possible simply, e.g., if methylated and unmethylated oligonucleotides are provided with different labels or if they are bound spatially separately on a solid phase (Fig. 2). The person skilled in the art knows how to obtain information on enzymes that can be used in this embodiment. In particular, the REBASE database (<http://rebase.neb.com/>) offers a great deal of information on hemi-methylation-sensitive restriction enzymes. The use of the following enzymes is preferred: AcsII; Adel; AscI; HincPI; ClaI; EciI; HincP1I; Hpy99I; NruI; RsrII; Sall. The restriction sites of these enzymes are listed in the Appendix. Reaction conditions for the enzyme reaction are prior art and can be taken, e.g., from the protocols supplied by the manufacturers.

In another embodiment of the method according to the invention, enzymes are utilized which preferably cleave unmethylated DNA as opposed to hemi-methylated and homo-methylated DNA. Then the use of unmethylated oligonucleotides is necessary (or additionally the application of methylated oligonucleotides for quantification). More detailed information on the enzymes that can be utilized is available from the above-named source.

As long as corresponding enzymes are available, it is in principle also conceivable to work with enzymes which do not cleave unmethylated DNA, but do cleave hemi-

methyated and homo-methyated DNA, or which do not cleave unmethyated and hemi-methyated DNA, but do cleave homo-methyated DNA.

It is obvious that biologically active fragments or modifications of the enzymes can also be utilized for the method according to the invention. With increasing success of the enzyme design, the use of enzymes constructed especially for the purposes of this invention are also conceivable.

According to the invention, several different restriction enzymes can also be utilized simultaneously or sequentially in combination with different oligonucleotides, in order to investigate the methylation state of several different cytosine positions.

The detection is conducted in the third step of the method according to the invention. This step is carried out according to experimental approaches in the prior art. If labeled oligonucleotides are utilized, then the labels either of the uncleaved oligonucleotides or of the restriction fragments can be detected. When a solid phase is used, it is also possible to detect restriction fragments which are found in solution, or fragments which are bound to the solid phase (e.g., with the use of a quencher). By coupling the oligonucleotides to a sensitive surface, the chemical or physical properties that change due to the restriction are measured.

Then in the fourth step, the methylation status of the DNA is concluded from the detected signal and the proportion of methyated DNA is determined.

If disease-specific cytosine positions are investigated, then the method according to the invention is particularly suitable also for the diagnosis of cancer disorders or other diseases associated with a change of methylation status. These include, among others, CNS malfunctions; symptoms of aggression or behavioral disturbances; clinical, psychological and social consequences of brain damage; psychotic disturbances and personality disorders; dementia and/or associated syndromes; cardiovascular disease, malfunction and damage; malfunction, damage or disease of the gastrointestinal tract; malfunction, damage or disease of the respiratory system; lesion, inflammation, infection, immunity and/or convalescence; malfunction, damage or disease of the body

as a consequence of an abnormality in the development process; malfunction, damage or disease of the skin, the muscles, the connective tissue or the bones; endocrine and metabolic malfunction, damage or disease; headaches or sexual malfunction. The method according to the invention is also suitable for predicting undesired drug effects and for distinguishing cell types or tissues or for investigating cell differentiation.

The subject of the invention is also the use of hemi-methylation-sensitive restriction enzymes for the methylation analysis and for the detection of the above-named diseases associated with a change of methylation status, in particular the use of the enzymes: AcsII; Adel; AscI; HinfI; ClaI; EcoI; HinfI; Hpy99I; NruI; RsrII; Sall for the above-named purposes.

A preferred embodiment of the invention is comprised of a test strip, on which oligonucleotides with a different methylation status and/or a different sequence are immobilized. This test strip is hybridized with the DNA to be investigated in a temperature-controlled mini-chamber. Restriction and detection are then performed in one step, e.g., in a cuvette in which the absorption spectra of the dyes used are measured (Fig. 3). In another preferred embodiment, the restriction fragments diffuse to another phase and therein lead to detectable secondary reactions.

The subject of the present invention is also a kit, comprised of different, immobilized oligonucleotides, at least one hemi-methylation-sensitive restriction enzyme and the necessary restriction buffers.

### Application example

The following example will illustrate the application of the method according to the invention for characterizing tumors. A tumor develops into two different types (A and B), each of which requires a different treatment. The two types, however, are not to be diagnosed on the basis of morphological features alone without anything further; they are distinguished, however, by their methylation status: a CpG base pair, which lies within the base sequence GCGC in the middle of a known sequence context, is methylated in tumor type A, whereas it is present unmethylated in type B. For



investigation, the DNA from the tumor tissue is extracted with a commercially available kit. A thermal denaturation of the DNA and a subsequent hybridization with an exact 1:1 mixture of the two synthetic oligomers C and D are performed. These latter oligomers possess the same base sequence (complementary to the DNA to be investigated), but are distinguished by their methylation status. The CpG which corresponds to the CpG whose methylation is to be determined is unmethylated in C, but methylated in D. C and D bear different fluorescent dye/quencher combinations, wherein the dye is found at one end of the oligonucleotide and a quencher, which prevents detection of the dye, is found at the other end. After the hybridization, a restriction of the DNA oligonucleotide hybrids that have formed is conducted. For this purpose, a large quantity of the hemi-methylation-sensitive restriction enzyme *HinPI* is added to the DNA. This enzyme cleaves hemi-methylated and unmethylated DNA, but not homo-methylated DNA in the sequence context GCGC. The increase in the two dyes (no longer blocked by the quencher) is measured over time during the progression of the reaction by means of a temperature-controlled fluorescence photometer. Only the combination of methylated DNA from tumor tissue with methylated oligomers, thus in this case A and D, leads to methylated DNA on both sides. This DNA is not cleaved by the restriction enzyme, so that a dye is not released. The proportion of methylated DNA in the investigated tumor specimen can be determined by the ratio of the released dye of the two oligonucleotides D and C. This value permits information to be obtained on the type of tumor and thus an optimal treatment.

#### Brief description of the figures

Fig. 1 shows a preferred embodiment of the invention with the use of a dye/quencher pair. In this case, a methylated oligonucleotide is bound to a solid phase. The oligonucleotide bears a dye (pentagon) and a quencher (square). The DNA to be investigated is hybridized to the oligonucleotides. Then it is reacted with a restriction enzyme. If the DNA to be investigated is unmethylated, then the hybrid is cleaved. Dye and quencher are separated and a signal can be detected.

Fig. 2 shows a preferred embodiment of the invention. In this case, two different types

of oligonucleotides are used. Both oligonucleotides have the same base sequence. They are, of course, distinguished by their methylation status and bear different dyes (circle and pentagon, respectively). The DNA to be investigated is hybridized to the oligonucleotides. Then a restriction is conducted. The degree of methylation of the specimen (M) can be determined from the ratio of the dye signals.

Fig. 3 shows schematically a preferred embodiment of the invention. In this case a tumor specimen is investigated for its methylation status by means of a test strip. For this purpose, the DNA extracted from the specimen is hybridized to oligonucleotides fixed on a test strip (step 1). Then a restriction is conducted with a hemi-methylation-sensitive restriction enzyme. (step 2). Different color patterns (A, B and C) result, each time depending on the methylation status of the DNA to be investigated (step 3). It is known from earlier experiments which methylation patterns are associated with specific types of tumor (4). Information for successful tumor therapy can be derived by comparison with these data.

#### Appendix: Hemi-methylation-sensitive restriction enzymes

The enzymes listed below show a selection of possible enzymes that can be used in the method according to the invention. The restriction sites are taken from the REBASE database. The conformations presented on the left side are cleaved. The restriction is retarded in the center and completely blocked on the right side.

Table 1

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 ABC11

CCC  
GCG  
n5

n5  
CCG  
GCG

n5  
CCG  
GCG  
n5

n5  
CCG  
GCG

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 ADe1

n5  
CACNNHGTG  
GTGNNHCA

n5  
CACNNHGTG  
GTGNNHCA  
n5

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Ascl

n5  
G G C G C G C C  
C C G C G C G G

n5  
G G C G C G C C  
C C G C G C G G  
n5

n5  
G G C G C G C C  
C C G C G C G G  
n5

n5 n5  
G G C G C G C C  
C C G C G C G G  
n5 n5

n5  
G G C G C G C C  
C C G C G C G G

n5  
G G C G C G C C  
C C G C G C G G  
n5

n5  
G G C G C G C C  
C C G C G C G G  
n5

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HindPI

n5  
G C G C  
C G C G

n5  
G C G C  
C G C G  
n5

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clai

n5  
A T C G A T  
T A G C T A

n5  
A T C G A T  
T A G C T A  
n5

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EC11

GGCGGA  
CCGCCCT  
n5

GGCGGA  
CCGCCCT  
n5

n5  
GGCGGA  
CCGCCCT

n5  
GGCGGA  
CCGCCCT  
n5

n5  
GGCGGA  
CCGCCCT  
n5 n5

n5  
GGCGGA  
CCGCCCT  
n5

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H1nPII

n5  
GGCG  
CGCG

n5  
GGCG  
CGCG  
n5

n5  
GGCG  
CGCG

n5  
GGCG  
CGCG  
n5

n5  
GGCG  
CGCG  
n5

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Hpy99I

n5  
CGWCG  
GCWGC

n5  
CGWCG  
GCWGC  
n5

n5 n5  
CGWCG  
GCWGC  
n5 n5

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 RruI

m5  
 TCGCGA  
 AGCGCT

n5  
 TCGCGA  
 AGCGCT  
 m5

n5 m5  
 TCGCGA  
 AGCGCT  
 m5 m5

m5  
 TCGCGA  
 AGCGCT

m5  
 TCGCGA  
 AGCGCT  
 m5

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 RsrII

m5  
 CGGWCCG  
 GCCWGGC

m5  
 CGGWCCG  
 GCCWGGC  
 m5

m5 m5  
 CGGWCCG  
 GCCWGGC  
 m5 m5

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 SalI

m5  
 GTCGAC  
 CAGCTG

n5  
 GTCGAC  
 CAGCTG  
 m5

n5 m5  
 GTCGAC  
 CAGCTG

m5  
 GTCGAC  
 CAGCTG

m5  
 GTCGAC  
 CAGCTG  
 m5

m5  
 GTCGAC  
 CAGCTG  
 m5